

Identification of feeding stimulants for Pacific coast wireworm by use of a filter paper assay (Coleoptera: Elateridae)

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ABSTRACT

Sugars and several plant essential oils were evaluated as feeding stimulants for larvae of Pacific coast wireworm, *Limonius canus* (Coleoptera: Elateridae). Compounds were evaluated by quantifying biting rates of wireworms on treated filter paper disks, modifying a method used previously in assays with *Agriotes* spp. wireworms. Independent counts of the same disk showed that the method led to repeatable estimates of biting rate. Higher rates of biting were obtained on filter paper disks if those disks had been treated with sucrose, fructose, glucose, maltose, and galactose, than if the disks were left untreated. Sucrose and fructose were more stimulatory than the other three sugars. Biting rates declined with decreasing concentrations of sugars in water. Combining a highly stimulatory sugar (sucrose) with certain plant essential oils in some cases led to non-additive (both synergistic and antagonistic) effects on biting rates. We discuss the possible role for this type of assay in developing insecticide-laced baits for attract-and-kill programs.

Key Words: *Limonius canus*, feeding assay, phagostimulants, synergism, plant essential oils

INTRODUCTION

Wireworms (Coleoptera: Elateridae) are important subterranean pests in a number of vegetable and grain crops worldwide. The Pacific coast wireworm, *Limonius canus* LeConte, inhabits irrigated soils of western North America, where it is a pest in potatoes, vegetables, and grain crops (Lane and Stone 1960). Grower difficulties in managing this and other wireworm pests can be attributed to a number of factors, including a shortage of chemicals effective against wireworms, lack of efficient monitoring tools, and incomplete understanding of wireworm basic biology (Jansson and Seal 1994).

Wireworm larvae are attracted to various types of food-based baits, including baits composed of germinating seed; wheat and rice flours; and rolled oats (Apablaza *et al.* 1977; Toba and Turner 1983; Horton and Landolt 2002). Historical success in drawing wireworms to food-based baits under field conditions has prompted efforts, beginning at least as early as the 1930s, to develop insecticide-laced baits for use in wireworm control (Lehman 1933; Woodworth 1938). Yet, almost 80 years following these first efforts, no toxicant-laced bait is commercially available for controlling wireworms in North

America. Difficulties in developing field-effective baits may often be due to wireworm behavior. Specifically, a bait that is highly attractive when free of a toxicant may become repellent to wireworms with addition of a toxicant (Lehman 1933; Woodworth 1938). Similar problems may affect how well coating of grain seed with insecticide protects germinating seed from wireworms. Protection of treated seed from wireworm damage may often be due to pre- or post-contact repellency of the insecticide rather than to actual kill of the pest (Long and Lilly 1958; van Herk and Vernon 2007; Vernon *et al.* 2009).

A long-term aim of our research program is to develop a toxicant-laced bait that can be used in an attract-and-kill program for managing *L. canus*. Ongoing trials with a food-based bait laced with an insecticide (formulation currently proprietary) have shown mixed results: rates of kill in laboratory trials are inconsistent, apparently due in part to antifeedant effects associated with presence of the toxicant (DRH pers. obs.). Improving bait palatability by the addition of feeding stimulants could lead to increased rates of kill if the stimulant prompts higher rates of feeding even in the presence of the toxicant.

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Compounds that elicit increased feeding by *Limoni* wireworms have yet to be specifically identified and assayed, and this has slowed our efforts to develop a consistently effective bait.

The objective of this study was to develop an assay method suitable for testing compounds as potential feeding stimulants for *L. canus*. Assays to determine whether certain compounds prompt feeding behaviour of subterranean insects generally involve application of test products to a substrate that allows feeding by the insect. We modified a filter paper assay developed over 50 years ago to examine biting response of *Agriotes* spp. wireworms (Thorpe *et al.* 1947; Crombie and Darrah 1947), and determined whether the method would be suitable for identifying compounds that elicit feeding of *L. canus*. We

then used this assay method to examine biting rates of *L. canus* in response to several sugars at different concentrations. Sugars have been shown to prompt feeding by a number of root-feeding insects (e.g., Thorpe *et al.* 1947; Allsopp 1992; Bernklau and Bjostad 2008), and may be stimulatory enough under some conditions to reduce the deterrent effects of otherwise repellent chemicals (Shields and Mitchell 1995; Bernklau *et al.* 2011). We next tested whether one particular sugar (sucrose) in combination with other plant compounds acted synergistically with those compounds in eliciting the biting response. We examined combinations of several plant essential oils with sucrose, as plant essential oils have been shown to both deter and elevate feeding by phytophagous insects (Tanton 1965; Klepzig and Schlyter 1999).

MATERIALS AND METHODS

Source of insects. Mid-sized to large larvae (1.2-1.4 cm in length) of *L. canus* were collected in spring from fields located near Yakima, WA and Hermiston, OR. The insects were collected by baiting with balls of moistened rolled oats (Horton and Landolt 2002). The Yakima field was fallow at the time of baiting, but had been planted to either wheat or potato crops in preceding years. Wireworms at the Hermiston site were collected along a fence line adjacent to potato or wheat crops. Larvae were stored in groups of 20-30 in 35 x 25 x 10 cm plastic tubs filled with moistened potting soil until they were used in the assays. Tubs were kept at room temperature (22-23°C). Small plugs of moistened rolled oats were added to each tub every 7-10 d, and removed after 48 h; otherwise, the larvae were kept unfed. Larvae were used within 1-3 weeks of having been collected. Assays were done in May and June of 2009 and 2012. Wireworms were discarded following each assay.

Quantification of biting response. Feeding response was assayed by quantifying biting marks of wireworms on treated filter paper disks (Thorpe *et al.* 1947; Crombie and Darrah 1947). Filter paper disks (Grade 413 qualitative filter paper, 5.5 cm diameter; VWR Scientific Products, West Chester, PA) were treated with individual compounds or with combinations of compounds (see below) and

presented to wireworms in either paired-choice or no-choice assays. The treated disks were placed in plastic petri dishes (14.5 cm diameter x 2 cm deep) filled with 200 ml of sand (Quikrete Premium Playground Sand, Quikrete, Atlanta, GA) moistened with 30 ml of tap water. In positioning a treated disk in the petri dish, we first filled each dish approximately one-quarter full with the moistened sand and placed the disk on the surface of the sand. The disk was then covered with enough additional sand to fill the petri dish approximately three-quarters full. Wireworms (see below for numbers used in each assay) were placed on the surface of the sand layer at the center of each petri dish and allowed to enter the soil. The insects were randomly assigned to treatments, to ensure that any variation in feeding rates associated with wireworm size was randomly allocated across the different treatments. The assays were conducted at room temperature. Petri dishes were kept covered to prevent the sand from drying.

After 24 h of exposure to wireworms, disks were examined for feeding damage. In studies with *Agriotes sputator* (L.), *Agriotes lineatus* (L.), and *Agriotes obscurus* (L.) (Thorpe *et al.* 1947; Crombie and Darrah 1947), the stimulatory response was quantified by counting bite marks on the disks. However, we found that it was often difficult to

determine where physically on a disk a given bite mark began and ended, which made this method somewhat subjective. This approach was especially problematic when highly stimulatory products were tested, as these products often led to large contiguous patches of damage on disks. Instead, we quantified biting rates on a disk by placing the disk on a light table, covering it with a transparent grid (0.5 x 0.5 cm squares), and then counting the number of squares in which any bite marks were observed (Fig. 1). Both sides of each disk were examined. Squares in which the feeding damage was observable on both sides of the filter paper disk were counted only once. Two people examined each disk, and an average of the two counts was used in the data summary and analyses. To examine repeatability of this method for estimating biting rates, correlation analysis was used to determine whether counts were consistent between the two people. The assessments of repeatability were done using the PROC CORR program in SAS (SAS Institute 2010).

(1) Sugars as feeding stimulants. Five sugars were assayed: D-sucrose, D-fructose, D-glucose, D-maltose, and D-galactose (Sigma-Aldrich, St. Louis, MO). Each sugar was tested at five concentrations in deionized water: 2% (2 g per 100 ml of water), 1%, 0.5%, 0.25%, and 0.125%. Each filter paper disk received 200 μ l of solution delivered by pipette, which led to quantities of sugar per disk between 4 mg (2% solutions) and 0.25 mg (0.125% solutions). Control disks received an equivalent amount of deionized water. Disks were assayed immediately following treatment. We used a choice test to examine

feeding stimulation, by pairing a treatment and control disk in our feeding arenas (as in Wensler and Dudzinski 1972). Paired disks were set 1 cm apart in the petri dish and buried in sand as described above. Each paired comparison was replicated 10 times. Three wireworms were used per feeding arena, and allowed to feed for 24 hrs.

For each pair of disks, we subtracted control results (number of grid squares showing feeding damage) from treatment disk results. Thus, large positive values indicate that the sugar was highly stimulatory, whereas values near zero indicate that damage was similar on sugar-free and sugar-treated disks. These arithmetic differences were then used in a two-way factorial analysis of variance to assess the effects of sugar type and sugar concentration on biting response. A Tukey-Kramer means separation test was used to compare sugars following a significant ANOVA. To test whether a particular sugar at a specific concentration was significantly stimulatory, we compared simple effects means (i.e., a specific sugar at a specific concentration) to a hypothesized value of zero, using a t-statistic. Thus, a mean found to be significantly larger than zero was evidence that the sugar at that particular concentration was stimulatory. Analyses were done with the PROC GLIMMIX program in SAS (SAS Institute 2010).

(2) Additive and non-additive effects of sucrose and plant essential oils. These trials were done to determine whether our filter paper assay could be used to demonstrate non-additive (synergism or antagonism) effects of plant essential oils if combined with a sugar.

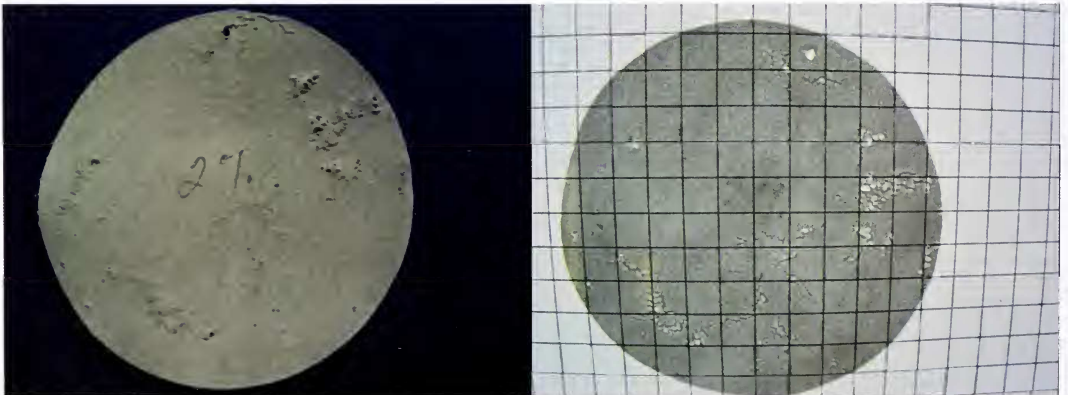


Figure 1. Sucrose-treated disk showing feeding damage (left photograph), and the same disk on light box showing grid (0.5 x 0.5 cm squares) used in quantifying damage (right photograph).

We examined five plant essential oils in the presence and absence of sucrose: lemon (*Citrus limon*), garlic (*Allium sativum*), winter savory (*Satureja montana*), cedarwood (*Juniperus virginiana*), and tea tree (*Melaleuca alternifolia*) (Herbal Advantage, Rogersville, MO; Mountain Rose Herbs, Eugene, OR). These compounds were chosen because preliminary trials suggested that a range of effects (synergistic to antagonistic) would be produced when the compounds were used in combination with a sugar. Sucrose was chosen for these trials because this sugar was found in our assays with sugars to elicit substantial rates of biting (see Results).

The literature of insect feeding trials is not always consistent in how synergism and antagonism are defined and demonstrated. We used an experimental design that allowed us to statistically demonstrate either of these two effects as the interaction term in a factorial analysis of variance. The design was a 2 x 2 (sucrose x plant oil) factorial experiment in which sucrose was at one of two levels (present vs. absent) and the plant essential oil of interest was at one of two levels (present vs. absent). Thus, unlike the previous trial with sugars, this assay was done using a no-choice design having (for a given plant oil) four possible treatments. A significant interaction term in the analysis of variance would be evidence of non-additive effects: i.e., biting rate in the combined sucrose + plant oil treatment was either higher (synergism) or lower (antagonism) than the sum of their separate effects.

All plant oils were diluted in solvent as 10 mg of the product in 100 ml of methylene chloride. Sucrose was diluted to 0.2% in deionized water. In preliminary trials, we found that wireworms often failed to feed on

disks that were free of both sucrose and the plant oil, which led to difficulties in conducting analysis of variance tests (due to variance assumptions of ANOVA). Therefore, we redefined our two sucrose levels (i.e., present vs. absent) as sucrose present (0.2%) versus sucrose highly dilute (0.02%), thus substituting an extremely dilute level of sucrose for our no-sucrose level. This highly dilute level of sucrose prompted some biting by wireworms, and this in turn allowed us to use ANOVA to examine results.

Filter paper disks were first treated with 200 μ l of the diluted plant oil in methylene chloride or with 200 μ l of methylene chloride (for those treatments in which plant oil was not present). Disks were allowed to dry, and then were treated with 200 μ l of the appropriate sucrose solution (either 0.2% or the highly dilute solution). The disks were immediately placed singly in moistened sand and petri dishes as described above for the sugar trials. A single wireworm was added to each petri dish and allowed to feed for 24 h. At the end of 24 h, biting rates (numbers of squares showing damage) were quantified for each disk using methods described above. We had 20 replicates of each treatment.

Number of squares showing damage was compared among treatments using ANOVA for a 2 x 2 factorial design. If the interaction term was significant, we examined interaction graphs to assess whether biting rates in the combination treatment were higher than expected under an additive model (synergism) or lower than expected under an additive model (antagonism), and used the PDIFF command in SAS to examine comparisons of simple effects means (e.g., plant oil effects separately at each level of sucrose).

RESULTS

(1) Sugars as feeding stimulants. Estimates of biting rates (= numbers of squares showing damage) were highly correlated between the first count and second count (Fig. 2; data shown only for the sucrose-treated disks), suggesting that our counting method provided an objective and quantifiable index of biting rates. We observed biting marks in virtually all replications, except at the most dilute rate (Fig. 2). All five sugars

prompted biting by *L. camus* (Fig. 3); each mean is the average of the arithmetic differences in grid squares showing damage, between the paired sugar-treated and control disks. Both concentration ($F_{4,225} = 11.8$, $P < 0.0001$) and type of sugar ($F_{4,225} = 28.9$, $P < 0.0001$) affected biting rates. The sugar x concentration term was non-significant ($P = 0.28$). A means separation test showed that sucrose was significantly more stimulatory

than fructose, and that both products prompted more biting than glucose, maltose, and galactose (Fig. 4; the latter three sugars were statistically the same in their effects). Stimulatory effects disappeared at concentrations of 0.125% for fructose, and at 0.5% for glucose, maltose, and galactose (assessed using *t*-tests to compare each mean in Fig. 3 to zero); all concentrations of sucrose were stimulatory.

(2) **Additive and non-additive effects of sucrose and plant essential oils.** Results with the five plant essential oils are shown as a series of interaction graphs (Fig. 5), in which (+) indicates presence of the compound and (-) indicates that the compound is absent (plant oil) or is at a highly dilute concentration (sucrose at 0.02%). Additive (Fig. 5A), synergistic (Fig. 5BC), and antagonistic (Fig. 5DE) effects were each observed. Winter savory elicited biting responses whether in the presence or absence of sucrose (main effects of plant oil: $F_{1,76} = 19.1$, $P < 0.0001$); sucrose also was highly stimulatory ($F_{1,76} = 100.6$, $P < 0.0001$). The effects of winter savory and sucrose were additive, as shown by the non-significant interaction term (sucrose \times plant oil: $F_{1,76} = 0.6$, $P = 0.44$) and the parallel lines in the interaction graph (Fig. 5A).

Two plant oils (tea tree and lemon) exhibited synergistic effects with sucrose, as shown by a significant interaction term (sucrose \times plant oil: tea tree - $F_{1,76} = 8.0$, $P = 0.006$; lemon - $F_{1,76} = 5.1$, $P = 0.026$) and the nonparallel lines in the interaction graphs (Fig. 5B and C). For both plant oils, addition of the plant compound to sucrose (-) disks did not cause an increase in biting rates (comparison of simple-effects means, plant oil (+) versus plant oil (-) at sucrose (-): tea tree - $t_{76} = 0.8$, $P = 0.44$; lemon - $t_{76} = 1.9$, $P = 0.06$). Conversely, addition of the plant oil to sucrose-treated disks did elicit higher rates of biting (plant oil (+) versus plant oil (-) at sucrose (+): tea tree - $t_{76} = 4.8$, $P < 0.0001$; lemon - $t_{76} = 5.1$, $P < 0.0001$).

Both cedarwood and garlic appeared to inhibit response of wireworms to presence of sucrose (Fig. 5D and E). The plant oil \times sucrose interaction was significant for both products (cedarwood: $F_{1,76} = 4.6$, $P = 0.035$; garlic: $F_{1,76} = 5.3$, $P = 0.025$). Addition of either plant oil to sucrose (-) disks failed to cause significant changes in biting response

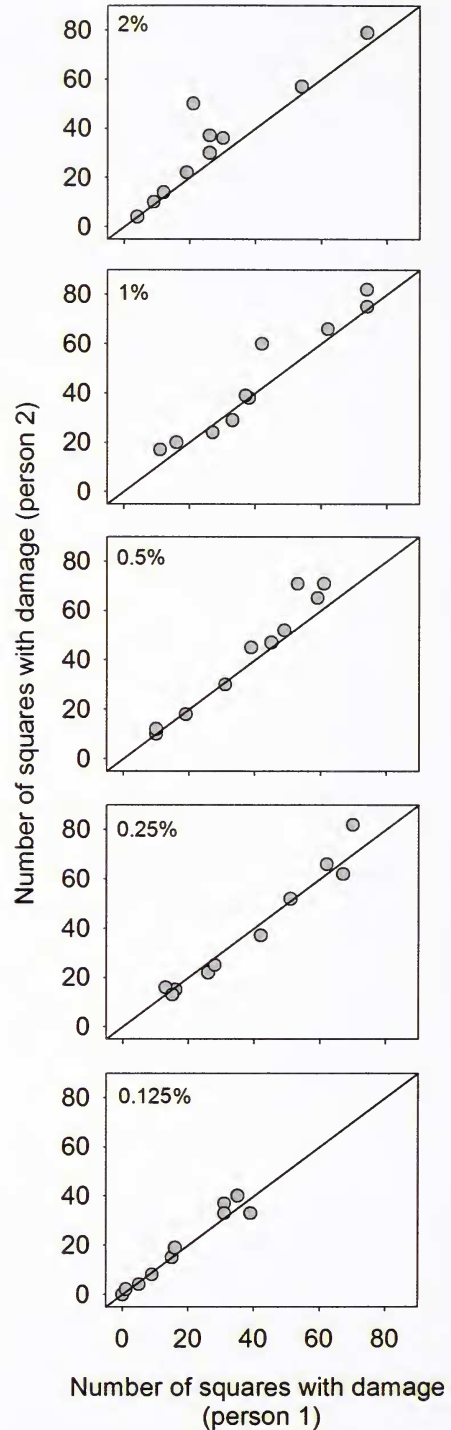


Figure 2. Scatter plots showing results for first (person 1) and second (person 2) estimates of damage; sucrose-treated disks ($N = 10$ disks per concentration). Correlations varied between 0.930 (2% concentration) and 0.982 (0.5% concentration).

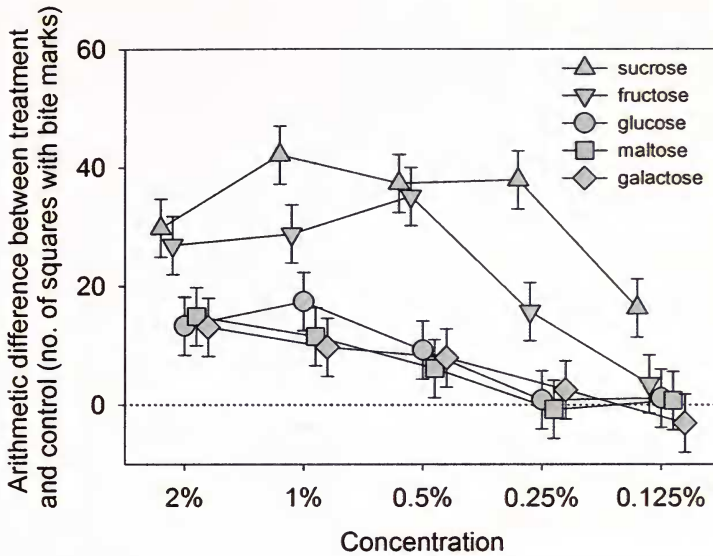


Figure 3. Mean (+ SEM) arithmetic difference between treatment (sugar) and control disks in number of squares showing feeding damage. Means are shown as a function of sugar concentration. Each mean is based upon 10 replicates.

(cedarwood: $t_6 = 0.8$, $P = 0.43$; garlic: $t_6 = 0.7$, $P = 0.52$). In contrast, adding either plant oil to the sucrose (+) disks actually led to statistically significant drops in biting rates

compared to rates seen on the sucrose (+) treatment (cedarwood: $t_6 = 2.2$, $P = 0.029$; garlic: $t_6 = 2.6$, $P = 0.011$).

DISCUSSION

The plant-associated cues that mediate feeding by wireworms or other subterranean insects are often inadequately known, in large part due to difficulties in studying these insects (Johnson and Gregory 2006; Johnson and Nielson 2012). This shortcoming may be especially pronounced for generalist species such as *L. canus*, given that its generalized feeding habits provide no obvious clues as to what plant compounds might elicit feeding. Several different approaches have been used to screen compounds as potential feeding stimulants or deterrents for either generalist or specialist root-feeders, most of which comprise an analysis of feeding or biting activity by the insect on a substrate that has been treated with the compound of interest. Substrates used in these assays have been quite diverse, and include at a minimum products such as filter paper disks (Thorpe *et al.* 1947; Wensler and Dudzinski 1972; Bernklau and Bjostad 2005), cellulose membrane disks (Ladd 1988; Allsopp 1992), thin sections of potato tuber (Villani and

Gould 1985), pith wafers (Thomas and White 1971), or agar (Tanton 1965). The assay developed here provided a repeatable means for estimating biting response of *L. canus* on treated filter paper disks.

Cues that prompt feeding by root-feeding Coleoptera often include any of several sugars (Chrysomelidae: Bernklau and Bjostad 2008; Scarabaeidae: Wensler and Dudzinski 1972, Ladd 1988, Allsopp 1992; and Elateridae: Thorpe *et al.* 1947, Crombie and Darrah 1947). Indeed, in a review of subterranean insects and their interactions with host plants, Johnson and Gregory (2006) showed that 48% of the chemical compounds shown to stimulate feeding by root-feeding insects were sugars. Thorpe *et al.* (1947) showed that the wireworms *Agriotes lineatus*, *A. sputator*, and *A. obscurus* were stimulated to bite filter paper disks if those disks had been treated with a sugar. Varietal differences in susceptibility of potato tubers to wireworm feeding are affected in part by levels of sugars in the tubers (Olsson and Jonasson 1995).

Here, we showed that biting of filter paper disks by *L. camus* was induced by any of five sugars, with sucrose and fructose being the most stimulatory (Fig. 3). Intensity of feeding, as estimated by counting bite marks, showed a decline with decreasing concentration of sugar in the solutions, to the extent that highly dilute concentrations of most products were not stimulatory (Fig. 3).

Plant compounds may interact either positively or negatively to affect feeding rates of phytophagous insects (Hsiao and Fraenkel 1968; Shanks and Doss 1987). Sugars have been shown to act synergistically with other (non-sugar) compounds in eliciting feeding behavior by above-ground and below-ground phytophagous insects (Crombie and Darrah 1947; Shanks and Doss 1987; Bartlet *et al.* 1994). Our assays with plant essential oils in combination with sucrose demonstrated any of three effects, depending upon the plant oil: additive, synergistic, and antagonistic. The exact mechanisms leading to these results are not clear, but could have included both gustation and olfaction. Volatiles from plant

essential oils are known to affect both short- and long-distance attraction and aversion responses of phytophagous insects (Landolt *et al.* 1999; Robacker 2007; Youssef *et al.* 2009). Similarly, gustatory signals from plant essential oils may inhibit or elicit feeding response (Tanton 1965; Klepzig and Schlyter 1999). Thus, the additive or synergistic effects observed here between sucrose and tea tree or sucrose and lemon theoretically could have been the result of either of two processes: (1) the plant essential oil acted as an additional feeding stimulant; or, (2) the plant oil acted as an olfactory cue that attracted the wireworm to the treated disk, and biting was then elicited by the sucrose. Antagonistic effects (Fig. 5DE) could have been due to inhibition of sugar receptors by the second compound (Ishikawa *et al.* 1969) or because the plant essential oil was modestly repellent (e.g., van Herk *et al.* 2010) and slowed how rapidly wireworms approached the sucrose-treated disks.

Historical efforts to use insecticide-laced baits for controlling wireworms have often

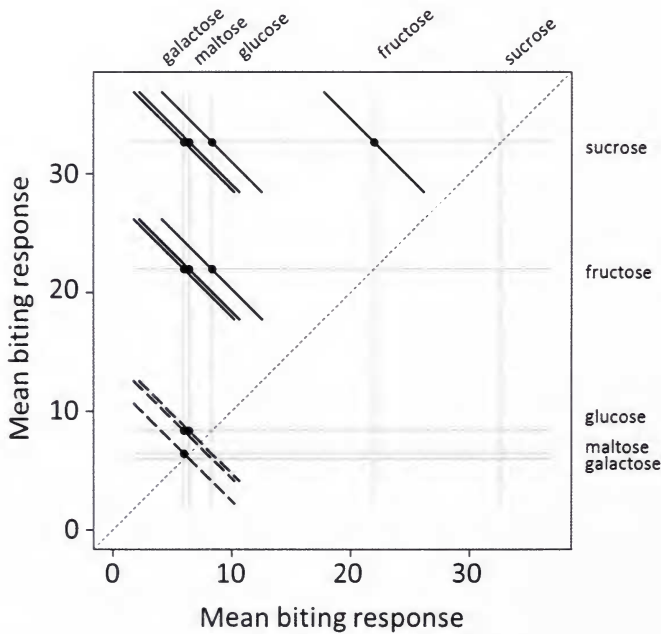


Figure 4. Diffogram showing results of Tukey-Kramer test for separating sugar means. Diagonal, upward sloping line depicts equality. Each solid circle shows joint location of two sugar means; the associated solid or dashed lines show confidence intervals for treatment differences (Tukey-adjusted). A confidence interval that intersects the equality line indicates that those two means are not statistically different (shown as dashed lines); a confidence interval that fails to intersect the equality line indicates that those two means are statistically different (shown as solid lines).

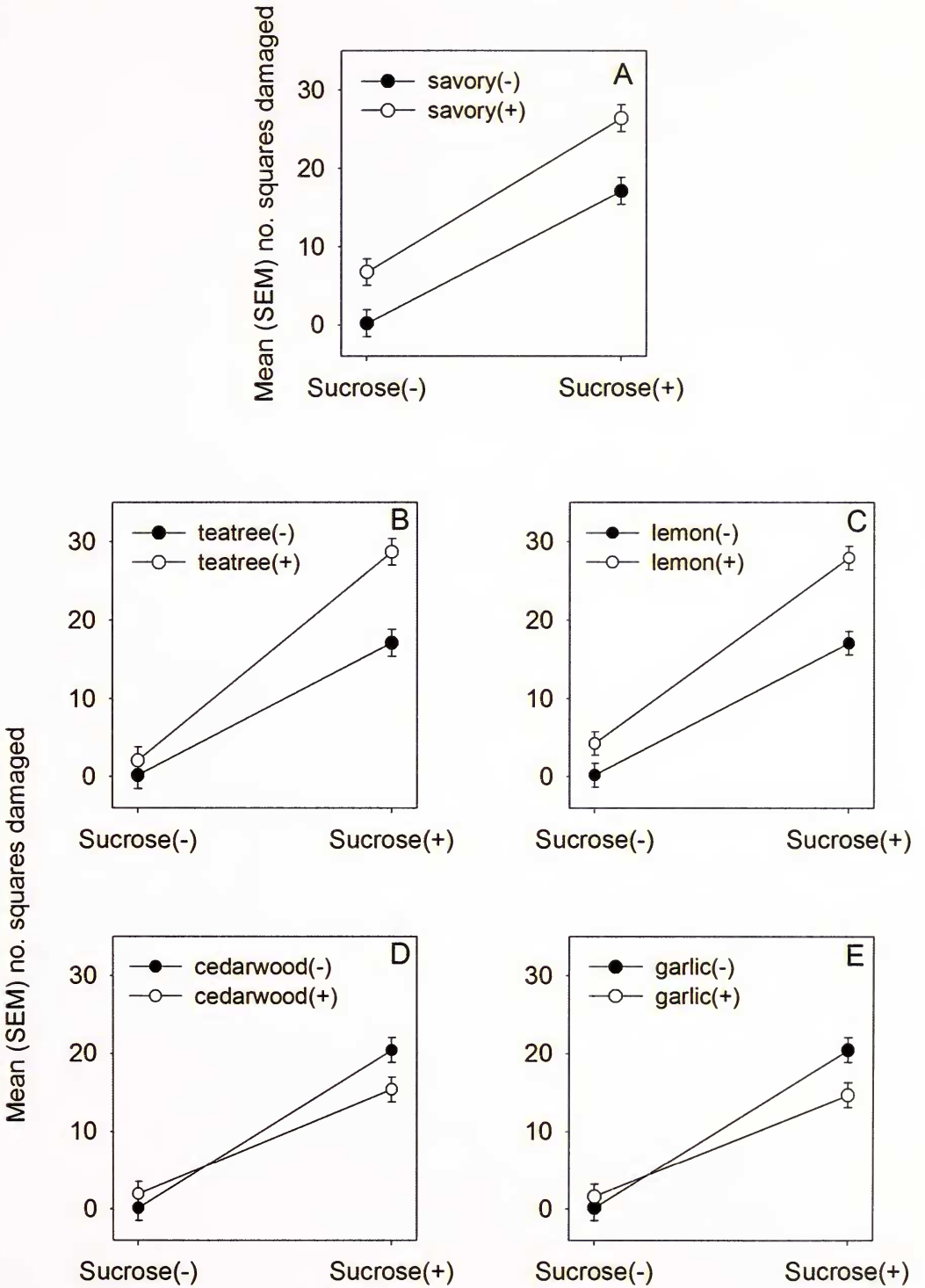


Figure 5. Interaction graphs showing the separate and combined effects of sucrose and plant essential oils on damage to filter paper disks. A: an additive effect; B and C: synergistic effects; D and E: antagonistic effects. Each mean based upon 20 replicates.

been unsuccessful (Lehman 1933; Woodworth 1938), apparently due to antifeedant or repellent effects of the toxicant (see also Long and Lilly 1958; van Herk and Vernon 2007). Addition of an appropriate phagostimulant could theoretically lead to improved rates of kill. For example, in trials with western corn rootworm larvae, *Diabrotica virgifera* LeConte (Coleoptera: Chrysomelidae), addition of a phagostimulant to insecticide-treated disks of filter paper led to higher rates of feeding on disks and increased kill of larvae than found in the absence of the

phagostimulant (Bernklau and Bjostad 2005; Bernklau *et al.* 2011). The studies summarized here provide a simple tool for screening of compounds for gustatory effects, including non-additive effects elicited by combinations of products, with possible longer-term benefits of developing a palatable bait. Additional compounds such as proteins or fatty acids shown in filter paper assays to elicit biting responses of other wireworm species (Thorpe *et al.* 1947) also merit attention for effects on *Limoni* spp. wireworms.

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